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(54) **DIAGNOSTIC AND THERAPEUTIC APPLICATION OF CTL AND NK FUNCTIONALLY SELECTED CELLS**

DIAGNOSTISCHE UND THERAPEUTISCHE ANWENDUNG FUNKTIONELL SELEKTIONIERTER
CTL- UND NK-ZELLEN

APPLICATION DIAGNOSTIQUE ET THÉRAPEUTIQUE DE CELLULES CTL ET NK
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Description

FIELD OF THE INVENTION

[0001] The invention proposes a novel therapeutic and diagnostic methodology based on the use of effector cells (for example CTL and NK cells) selected for being able to induce lysis in target cells. In particular, the invention teaches how to select strains of effector cells of the immune system based on their lytic properties. It also teaches how diagnostic procedures for checking the activity of therapeutic vaccines can be improved in view of what shown. Finally, it shows how cell therapies used at present can be improved by using this approach.

[0002] It is to be noted that here and below, by the terms "effector cell/s" or, more generally, by the terms "effector/s" is understood to mean a particle (cell or generic microorganism) which has an effect on a "target" particle, in turn defined as a cell or generic microorganism on which the modification induced by the effector is expressed.

STATE OF THE ART

[0003] Some important operating properties of the immune system are determined by the action of cell groups considered as "rare". In this context, by "rare" is understood to mean a subpopulation of cells having a frequency of a part per thousand or less within a known subpopulation. It is known, for example, that the progression of diseases such as the oncologic ones can be contrasted by a subgroup of immune system cells having the property of inducing lysis in target cells having opportune characteristics. It is equally known that such effector cells are rare, so this action is not much effective if not stimulated by opportune therapeutic means.

[0004] In the therapeutic field, CTL and NK cells are now isolated through a pre-purification, combined with a clonal selection by dilution "at the limit" (10). These strategies, even though they are valid and have met applicative successes, are complex, lengthy and expensive. Furthermore, the strategy of clonal selection not always can be activated, since in many cases characteristics of receptors of target cells (for example belonging to a tumour) to be attacked are not known or only partly known.

[0005] In this regard,

1. it is known that in cases in which tumour-specific peptides are known, pre-selection of populations of tumour-specific CTLs is possible and already described in therapeutic protocols (11);
2. it is known that in most cases, neoplastic cell-specific CTLs are present in the patient in the order of 1/1000-10,000 (12);
3. it is possible to isolate, with immunological techniques, populations of CTL within which tumour-specific CTLs can be identified (13);
4. single CTLs can be expanded in vitro for giving

rise to homogeneous populations utilizable for following biochemical analyses (14) and, if expanded in a sufficient way, for the immunotherapy of tumours, should they maintain specific for malignant cells (15):

5. strategies completely similar to those of CTL isolation can be applied to other effector cells capable of inducing lysis in target cells;

6. the use of CTLs (and analogous effector cells) in immunotherapy is not restricted to the treatment of tumours but can concern other pathologies, among which viral pathologies (for example AIDS) and pathologies due to a bacterial infection.

[0006] Considerations carried out for CTLs are also extended for NK cells, above all as far as the use of the same is attained, if particularly active in inducing lysis in tumor cells (16).

[0007] Altomare L. et al. (2003) discloses a printed circuit board device able to generate dielectrophoresis-based, software-controlled cages allowing to move tumour cells "entrapped" in the cages.

[0008] Fuchs AB. et al. (2006) discloses a microelectronic chip integrating an array of electrodes and sensors allowing individual and parallel single cell manipulation of samples of cells while maintaining viability and proliferation.

[0009] Rickinson AB. (1995) discloses the harvest of CTLs from a patient and the reinfusion in the patient after reactivation *in vitro*.

[0010] Schoell WMJ. et al. (1999) discloses a method for generating *in vitro* HPV-16 E7 peptide-specific cytotoxic T lymphocytes (CTLs) from PBMCs of healthy subjects for future adoptive immunotherapy of cervical cancer.

[0011] In the light of these considerations, a method which allows to select in a reasonable time and in an efficient way highly lytic CTL clones with respect of target cells is of a great importance in a therapeutic ambit.

SUMMARY OF THE INVENTION

[0012] The present invention teaches a method for the functional selection of immune system cells by means of real-time monitoring and quantification of the lysis of target cells (for example tumor cells) mediated by T-cytotoxic lymphocytes (CTL) (1) and other cells capable of inducing lysis in opportune cell targets (typically Natural Killer cells NK) (2). Such method is based on an innovative analytical assay of CTLs activity and other lytic cells with higher capacities than those presently available. Therefore, the present invention has immediate applications in the diagnostic sector. Furthermore, such method is proposed for an effective strategy for the isolation of clones of effector cells (typically CTLs or NKs) having high activity. Therefore, the present invention has important applications in the therapeutic sector.

[0013] In particular, the invention relates to a method

for the selection of immune system cells previously collected from a human being, useful in particular for the obtainment of important information to the diagnostic/prognostic check of the disease state of a neoplastic patient, including the steps of: a) let the immune system cells previously collected interact with respective target particles a modification of which, due to said interaction, is an index of a desired property of the immune system cells; b) checking the effect of the interaction on the target particles; and c) selecting, amongst immune system cells that have undergone the interaction with the target particles, those that have induced the modification in the target particles, therefore acting, towards the same, as effector cells.

[0014] Furthermore, the method according to the invention can also include the step of expanding immune system cells which have been selected at the end of step c), in order to create clones of antitumor effectors selected upstream by particular effectiveness of the lytic mechanism from heterogeneous populations (circulating or "standing" in draining lymphatic districts) of mononuclear cells.

[0015] According to a further aspect of the invention, the expansion step is performed by successive stages, carrying out between a stage and the following one a re-selection of effectors with optimal characteristics during the *ex vivo* amplification of possibly instable clonal populations, in particular repeating the steps a), b) and c) on at least one said possibly instable clonal population.

[0016] The invention further relates to the use of the above method for the preparation of a drug including immune system cells which have been selected through the steps a), b) and c) and/or their clones in a carrier suitable for an *in vivo* re-infusion with therapeutic purposes, possibly after stimulation with dendritic cells.

[0017] Finally, the invention relates to the use of a miniaturized device which allows the manipulation of effector cells and target cells without altering the biological activity thereof, in order to carry out the selection of effector cells and for the preparation of a drug of the type above indicated, as well as the use of selected and expanded lytic effector cells for the preparation of a drug for the treatment of pathologies

[0018] which can be treated by *in vivo* re-infusion of selected and expanded lytic effector cells, characterized in that the drug only includes effector cells previously selected and/or clones of effector cells previously selected all having a substantially comparable lytic activity.

BRIEF DESCRIPTION OF FIGURES

[0019]

Figure 1.A: CTLs displacement (3 CTLs: CTL1, CTL2, CTL3 indicated by arrows) directed to hit a target tumor cell (indicated by an arrow on the left). B: CTLs and target cell complex.

Figure 2. Interaction of CTLs (black arrows) and of

one target cell (white arrow) : the target cell is in an advanced lysis state.

Figure 3.A: lysis kinetics of target cells. B: non pre-activated CTLs.

Figure 4. A: calcein effect on lytic activity of CTLs measured through lysis % in a Cr51 release assay. In black bar graphs the specific lysis of target LCLs is reported (B35, loaded with EBV peptide), in the presence (on the left) or in the absence (on the right) of calcein; in white bar graphs, the control represented by LCLs unloaded with the peptide is reported. B. effect of the mannitol buffer on the lytic activity of CTLs (Cr51 release assay). In black bar graphs, the specific lysis of target LCLs is reported, in a mannitol-containing buffer (on the left) or in a standard buffer (RPMI; on the right); in white bar graphs, the control represented by LCLs unloaded with peptide is shown.

Figure 5. Detection of specific lysis of target LCLs loaded with the EBV peptide in a mannitol buffer, after DEP (dielectrophoresis) displacement and loading with calcein, on SmartSlide®. A-D panels: lysis of HPV-positive LCLs by HPV-specific CTLs has been detected after 10' and 20'; panels E-H: on the contrary, EBV-specific CTLs do not lyse LCLs unloaded with the peptide.

Figure 6. Reported data are the mean of three experiments wherein the decrease of the fluorescence signal (calcein) is detected over time, due to lysis of LCL-B35 loaded with EBV peptide, by specific CTLs after co-incubation in a standard buffer (RPMI) for 15' (panel B) or 30' (panel C). A parallel control detection (white bar graphs in A; empty symbols in C) shows that lysis is specific; in fact, it is absent if LCLs are not loaded with the EBV peptide; the decrease of the signal intensity over time is, in this case, minimum.

DETAILED DESCRIPTION

[0020] For the analysis of CTLs activity, the most currently used method is based on Cr51 release from lysated cells (3). Other methods, based on the release of non-radioactive europium (Eu3+) (4), the release of fluorescent markers (for example calcein) (5), the analysis of the esterase activity, the use of annexin V or beta-galactosidase, have recently been adjusted. Other methods based on impedance electronic analyses are also commercialised by Acea Biosciences, which allow a real-time check of lytic effects on cell aggregates induced by

[0021] These methodologies suffer from some important drawbacks. In some cases, they are based on the use of radioactive molecules (3) and always determine "average" results and not at a level of single cells (4, 5). When FACS (fluorescence-activated cell sorter) analysis (8) can be used, this requires a significant number of cells and complex and expensive instruments. In all these analyses, a real-time evaluation can not be effective.

[0022] In view of these considerations, a method allowing to monitor in a real-time and in effective way the cytotoxicity mediated by effector cells (for example CTLs) by extending such analysis at a level of single cell or highly lytic clones towards target cells, is certainly of a great importance in a diagnostic and therapeutic ambit.

[0023] In this regard,

1. it is known that CTL target cells can be subjected to staining using different supravital dyes, some of which can be released outside the cells if they are lysated and/or damaged (calcein (5) belongs to these supravital dyes);
2. it is known that CTL target cells can be subjected to staining using different supravital dyes which identify complex structures (mitochondria, nucleus, etc.) and which can not be released outside the cells, even if they are damaged (9);
3. opportune data processing programmes and imaging protocols can be used for the purpose of quantifying in an automated way the percentage of cells which manifest determined characteristics.

[0024] Concerning the real-time cell manipulation, the technology has recently provided some technologies. For example, tweezers lasers allow to create optical cages which can contain cells and, by moving the cages, contact cells to one another (17). It is moreover known that Lab-on-a-chip devices based on dielectrophoresis (DEP) can be very useful for displacing in a programmed way biological items (18-21). In particular, devices consisting of array-electrodes can be capable of manipulation single cells or single cell groupings.

[0025] An example of Lab-on-a-chip based on array-electrodes is shown in (21). At the base of the present invention there is the use of real-time methodologies for manipulation CTLs, NKs and target neoplastic cells and which are able to displace single cells as well, for the purpose of developing an effective method first for identification and then isolation of highly cytotoxic CTLs and therefore usable in treating neoplastic pathologies through immunotherapeutic strategies.

EXPERIMENTAL PROCEDURES

[0026] Lymphoblastoid cell lines (LCL) were obtained after infection of human B lymphocytes with the Epstein-Barr Virus (EBV) strain B95.8 (26). EBV-specific peptide HPVGEADYFEY (HPV), corresponding with aa 407-417 of EBNA1 protein was used for stimulation. Lymphocytes from peripheral blood (PBL) from a HLA-B35 donor were plated at a concentration of 3.5×10^6 cells per well in 24-well plates in a culture medium RPMI 1640, 10% FCS (Hyclone) and stimulated with HPV peptide (10 μ M). Cultures were again stimulated after 7 and 14 days and the medium was supplemented with 10 U/ml rIL-2 (Chiron). At days 14 and 21 cultures of T-cells were analyzed by CTL activity using appropriate cytotoxicity as-

says (51Cr-release) (3).

REMARKS ON OBTAINED RESULTS

[0027] Results of such study are shown in the examples reported in Figs. 1-6. Examples are given for illustrative purpose and are not intended to limit the scope of the invention in any way. In Fig. 1 the displacement of three CTLs (CTL1, CTL2, CTL3 indicated by arrows) directed to hit a single target tumor cell, by generating complexes shown in the right side of the figure, is reported, wherein CTLs appear indicated by CTL1, CTL2, CTL3 and arrows and the target cell is indicated on the left by an arrow.

[0028] We have surprisingly found that CTLs, displaced on a Lab-on-a-chip containing array electrodes can be contacted with neoplastic target cells and are capable to lysate them in a very quick time (varying from 8 to 20 minutes). If cells are marked with calcein, they are fluorescent if intact, losing the fluorescence if damaged by CTLs (Fig. 2 and Fig. 3). By carrying out double stainings using dyes for mitochondria or DNA, cells damaged by CTLs lose their fluorescence of calcein maintaining the one of specific dyes for mitochondria or DNA. Therefore, through a fluorescence microscope analysis (possibly using imaging programmes), it is possible to discriminate active CTLs from inactive CTLs. Non-selected CTLs do not show lytic activity on LCLs non pre-loaded with peptide.

[0029] From the other hand, as it has recently been described (22-25), cells manipulated by DEP (dielectrophoresis) maintain their phenotype and their differentiated features. According to the present invention, it has been noted that also the cytolytic activity and the detection of an optical signal (fluorescence) are not altered by this type of manipulation. This approach, therefore, is suitable for the immediate identification and following isolation and expansion of active CTL clones, as well as other cell populations which show a cytolytic activity towards determined cell targets.

[0030] In addition to optical imaging techniques used in this demonstration, it is known that impedance measurement techniques (6) allow to check the state of single cells with a high level of accuracy. The optical technique presented in this embodiment is therefore to be intended as merely exemplificative of a more general method.

[0031] Fig. 2 shows details of cells in an advanced lysis state after interaction with CTLs. In particular, in the right part of figure a lysated target cell by three CTLs (black arrows) can be observed (white narrow). Fig. 3 (panel A) shows lysis kinetics in CTLs/target cells aggregates. In B it is pointed out that non-preselected CTLs are not lytic. As it can be seen, the strategy allows to analyze lysis kinetics after a contact with CTLs and to identify CTL patterns with a high cytolytic activity.

[0032] Fig. 4A shows that a marking with calcein does not alter the lysis mechanism or the specific recognition of (LCL) HLA-B35 lymphocytes loaded with EBV peptide

from CTLs. In Fig. 4B there is also shown that neither dielectrophoresis nor mannitol-containing buffer have any effects on this mechanism, a result which is aligned with what has been checked by others for the gene expression profile (37) and the growth ability of K562 (35, 36), which do not result to be altered by dielectrophoresis.

[0033] The maintenance of the specificity of recognition reactions and lysis in the used experimental conditions (mannitol buffer and displacement through dielectrophoresis) has further been checked in experiments shown in figures 5 and 6. In figure 6 there is also shown kinetics of fluorescence intensity variation following to specific lysis of LCLs from CTLs, which can already be pointed out within the first 8-10 minutes from DEP (dielectrophoresis) treatment, also starting from a limited number of cells (no.= 25, 30, 20, 27). In conclusion, it has surprisingly been found that the specificity of the immune reaction remains preserved in this experimental context. In fact, it is absolutely not obvious that the immune reaction gives rise to a selective behaviour also in the presence of an extremely reduced number of cells. In particular, the environment in which this kind of manipulation takes place prevents amongst cells which, on the contrary, occur in all other methodologies. Despite this, the finding of the selectivity and specificity of the immune reaction opens up doors to an important series of teachings of this patent.

[0034] The fact that cytotoxic effectors (CTLs in the reported example, but also NK cells and other) can be enumerated *ex vivo* in a real-time and that this analysis is not based on "surrogate endpoints" (e.g. the expression of a surface specific marker of the effector, the release of a lymphokine, the expression of differentiation antigens, etc.) but rather on the online and real-time quantitative reading of the lytic effectiveness at a level of a single cell, is innovative and with a great importance. It is known, in fact, that not all T-lymphocytes which recognize a given antigen are equally lytic (2, 28) and that the percentage of mature phenotype-lymphocytes increases during vaccinations (29). It is expected that the use of the direct cytotoxicity as an endpoint greatly correlates with the clinical prognosis with respect to the use of surrogate endpoints, and allows a more accurate monitoring of the effectiveness of the vaccine preparations in inducing a specific immunity. Furthermore, the presence of antitumour T-lymphocytes is often observed in concomitance with a relapse and return of a disease also in patients not recently vaccinated (30).

[0035] Being the technique of cell recovery after the manipulation (33) a known art, this procedure has therapeutic involvements. The isolation and the recovery of effector cells with a high lytic activity allows the "in vitro" cultivation of highly selected cells, with the aim of carrying out biochemical studies and expanding the selected populations. Both strategies have deep implications in immunotherapy of tumours.

[0036] In fact, the isolation of high selectivity cytotoxic effectors from heterogeneous populations (circulating or

"standing" in draining lymphatic districts) of mononuclear cells allows (a) the clonal expansion of antitumor effectors selected upstream, for the particular effectiveness of the lytic mechanism; (b) the re-selection of effectors during the *ex vivo* amplification of possibly instable clonal populations. Technologies for *in vivo* re-infusion for therapeutic purposes, possibly after stimulation with dendritic cells, are available (31). The effectiveness of local and/or systemic re-infusion of these effectors, and the take of the autologous infusion can also be monitored over time and correlated with the immune state and the course of the disease (31).

[0037] The possibility of expanding *in vitro* highly lytic cells, changing, if necessary, the phenotype and re-infusing them in patients suffering from neoplasia (adoptive-cell-transfer therapy) has been a subject of multiple studies (28-30, 32). In this experimental strategy, the rapid identification of effector cells with a high lytic activity and the ability of generating cell loadings having a therapeutic importance however maintaining the gene stability is still a limiting factor.

[0038] The strategy subject of this patent is compatible with a lowering of times and an improvement of the selection procedure of cells with high lytic activity, facilitating, from one side, the identification of target tumor antigens, from the other side the expansion of cells usable in immunotherapy. If, by way of example, the method suggested in (32) is considered for obtaining a functional selection of useful strains of cells potentially having an induction activity of lysis in target cells, the improvement in the selection quality obtained by the present methodology is better appreciated. The technique in (32) starts from the observation that there are cell strains, TILs or Tumor Infiltrating Lymphocytes, capable of infiltrating in the tumor tissue and exerting a lytic activity towards carcinogenic cells. Also in this case, the number of TILs found in patients is lower than the required for obtaining a disease remission. The protocol can then be summarized in the following steps: 1) obtaining of biopsy from the patient, 2) treatment of the tissue so as to allow the *in vitro* infusion of opportune nutritive and growth factors for the desired cells, 3) after the growth step has ended, purification of cell strains of TILs and removal of tumor cells, 4) re-infusion in patients of the cells thus obtained. It is known that only a fraction of TILs obtained in this way shows lytic activities and therefore, as the quantity of cells re-infused in the patient for limiting undesired side effects can not be exceeded, the therapy has a restricted effectiveness. The methodology proposed in this patent reduces in a significant way the limitations of the one discussed herein as 1) the selection of effector cells can be extended at cell lines also existing in the peripheral blood and is not limited to TILs; 2) the functional selection produces a set of strains whose lytic ability is known; 3) it allows to improve the expansion procedure of these cell lines owing to the possibility of monitoring in different expansion steps the stability of the concerned cell lines; 4) it reduces the cell loading required for the therapy and

therefore, on the same material re-infused in the patient, provides a more aimed action. The point 3) improves in a significant way the state of the art relating to the expansion of CTL-type cell lines which are known to change the lytic phenotype after a certain number of expansions. In the extreme case in which the availability of a single cell having the desired lytic features is downstream the selection procedures and it is necessary to reach cell loadings having therapeutic valences of the order of some millions of cells, about 25 or 30 expansion cycles are required.

[0039] This number of cycles is such not to maintain the genic stability of the involved cells, so that subpopulations having a different lytic phenotype are generated. The presented invention teaches how to solve this problem. In fact, the functional selection of cells can be repeated at different stages of the growth procedure and for example when, after 10 or 12 growth cycles, some thousand of cells are available. The purification of the cell line can be restored through an extended application of the functional selection above disclosed. It is in fact possible to maintain target cell lines obtained by the biopsy and repeat the suggested method for eliminating lytic cells having an undesired phenotype. The elimination of lytic cells at this expansion level is consistent with selection technologies based on electronic techniques and therefore pure lines are again obtained. A further expansion for a number of cycles equal to 10 or 12 produces a number of lytic cells having adequate numbers for therapeutic applications. Even if at this point the cell number is such to prevent an examination at a single cell level of the stability of the lytic phenotype, the number of growth cycles without control of the phenotype is now very reduced and therefore cells can be considered as proper reproductions of the original ones.

[0040] The invention further contemplates the use of any device suitable for cell manipulations which do not alter the biological activity of effector cells (in this case CTLs).

[0041] From the other hand, the invention relates to the isolation of each type of cell (including NK cells) capable of lysating target cells.

[0042] Finally, the invention is applied to all pathologies for which the cytolytic activity of effector cells (NKs or CTLs) can then be mainly directed, as above observed, to neoplastic pathologies, infective pathologies, autoimmune pathologies and inflammatory pathologies of acute and chronic type.

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Claims

1. Method for the selection of desired immune system cells having effector/target lytic properties among a population of immune system cells, including the steps of:

- a) letting the immune system cells previously collected from a human being interact with cells, in particular tumor cells and microorganisms;
- b) checking, the effect of the interaction in step a);
- c) selecting, amongst immune system cells that have interacted with the target cells, in particular tumor cells and microorganisms, only those immune system cells that have shown effector/target lytic properties;

characterised in that steps a), b) and c) are carried out by displacing single cells by means of a miniaturized device which allows real-time manipulation of single effector cells without altering the cytolytic activity thereof.

2. Method according to claim 1, wherein steps a), b) and c) are carried out so as to allow to quantify the presence of cytotoxic effectors, i.e. as T and NK cells and other, within the peripheral blood, inflammatory neoplastic infiltrates, draining regional lymph nodes and within any other site or accessible biological fluid of the human being from which the immune system cells were previously collected.

3. Method of determining a minimal residual disease in risk patients by measuring the cytolytic activity of antitumor T and NK effector cells according to the method of claims 1 and 2.

4. Method according to claim 3, wherein the cytolytic

activity is measured either by optical imaging techniques made possible by previously marking at least said target cells, in particular tumor cells and micro-organisms, or by impedance measurement techniques.

5. Method according to any one of claims 1 to 4, further including a step of collecting and expanding immune system cells which have been selected at the end of the step c) in order to create clones of antitumor effectors selected upstream for the particular effectiveness of the lytic mechanism.
6. Method according to claim 5, wherein said expansion step is carried out by *ex vivo* amplification and re-selection of effectors with optimal features, so as to eliminate instable clonal populations, by repeating steps a), b) and c) on the expanded immune system cells which have been selected at the end of the step c).
7. Use of the method according to any of claims 1 to 6 for the preparation of a drug including immune system cells which have been selected through said steps a), b) and c) and/or of their clones in a carrier suitable for an *in vivo* re-infusion for therapeutic purposes, possibly after a stimulation with dendritic cells.
8. Use according to claim 7, for the preparation of a drug for the treatment of a pathology which can be treated with infusion of selected and expanded lytic effector cells.

Patentansprüche

1. Verfahren zur Auswahl von gewünschten Zellen des Immunsystems mit lytischen Effektor-/Target-Eigenschaften in einer Population von Zellen des Immunsystems, beinhaltend die folgenden Schritte:

- a) Interagieren lassen der zuvor von einem Menschen gesammelten Zellen des Immunsystems mit Zellen, insbesondere Tumorzellen und Mikroorganismen,
- b) Überprüfen der Wirkung der Interaktion in Schritt a),
- c) Auswahl, unter den Zellen des Immunsystems, welche mit den Zielzellen interagiert haben, insbesondere Tumorzellen und Mikroorganismen, lediglich der Zellen des Immunsystems, welche lytische Effektor-/Target-Eigenschaften gezeigt haben,

dadurch gekennzeichnet, dass Schritte a), b) und c) durch Verdrängung einzelner Zellen mittels einer miniaturisierten Vorrichtung durchgeführt werden,

welche Echtzeit-Manipulation einzelner Effektorzellen erlaubt, ohne deren zytolytische Aktivität zu ändern.

2. Verfahren gemäß Anspruch 1, wobei Schritte a), b) und c) derart durchgeführt werden, dass die Quantifizierung des Vorhandenseins zytotoxischer Effektoren, d.h. als T- und NK-Zellen und Anderen, innerhalb des peripheren Blutes, entzündlicher neoplastischer Infiltrate, Drainage von regionalen Lymphknoten und innerhalb jeder anderen Stelle oder zugänglicher biologischer Flüssigkeit des Menschen, von welchem die Zellen des Immunsystems vorher gesammelt wurden, ermöglicht wird.
3. Verfahren zur Bestimmung einer minimalen Resterkrankung in Risikopatienten durch Messung der zytolytischen Aktivität von Antitumor-T- und -NK-Effektorzellen gemäß des Verfahrens nach Anspruch 1 oder 2.
4. Verfahren gemäß Anspruch 3, wobei die zytolytische Aktivität entweder durch optische bildgebende Techniken gemessen wird, welche durch vorheriges Markieren von wenigstens der Zielzellen, insbesondere Tumorzellen und Mikroorganismen, ermöglicht wird, oder durch Impedanzmesstechniken.
5. Verfahren gemäß irgendeinem der Ansprüche 1 bis 4, ferner beinhaltend einen Schritt des Sammelns und Ausweitens von am Ende von Schritt c) gesammelten Zellen des Immunsystems, um Klone von für die bestimmte Effektivität des lytischen Mechanismus aufwärts gesammelten Antitumor-Effektoren zu generieren.
6. Verfahren gemäß Anspruch 6, wobei die Ausweitung durch *ex vivo*-Amplifizierung und Rückauswahl von Effektoren mit optimalen Eigenschaften derart durchgeführt wird, dass durch Wiederholung der Schritte a), b) und c) auf die ausgeweiteten Zellen des Immunsystems, welche am Ende von Schritt c) ausgewählt wurden, instabile Klonpopulationen eliminiert werden.
7. Verwendung des Verfahrens gemäß irgendeinem der Ansprüche 1 bis 6 zur Herstellung von Wirkstoff, welcher Zellen des Immunsystems enthält, welche durch die Schritte a), b) und c) ausgewählt wurden, und/oder von deren Klonen in einem für eine *in vivo*-Reinfusion zu therapeutischen Zwecken geeigneten Träger, möglicherweise nach Stimulation mit dendritischen Zellen.
8. Verwendung gemäß Anspruch 7, zur Herstellung eines Wirkstoffs zur Behandlung einer Pathologie, welche durch Infusion von ausgewählten und ausgeweiteten lytischen Effektorzellen behandelt wer-

den kann.

Revendications

1. Méthode pour la sélection de cellules souhaitées du système immunitaire dotées de propriétés effectrices/lytiques de la cible parmi une population de cellules du système immunitaire, incluant les étapes suivantes :

- a) l'étape consistant à laisser les cellules du système immunitaire prélevées auparavant à partir d'un être humain interagir avec des cellules, en particulier des cellules tumorales et des micro-organismes ;
- b) la vérification de l'effet de l'interaction dans l'étape a) ;
- c) la sélection, parmi les cellules du système immunitaire qui ont interagi avec les cellules cibles, en particulier les cellules tumorales et les micro-organismes, de seulement ces cellules du système immunitaire qui ont démontré des propriétés effectrices/lytiques de la cible ;

caractérisée en ce que les étapes a), b) et c) sont réalisées en déplaçant des cellules uniques au moyen d'un dispositif miniaturisé qui permet une manipulation en temps réel de cellules effectrices uniques sans modifier leur activité cytolytique.

2. Méthode selon la revendication 1, dans laquelle les étapes a), b) et c) sont réalisées de façon à permettre la quantification de la présence d'effecteurs cytotoxiques, c'est-à-dire comme les lymphocytes T et NK et d'autres, au sein du sang périphérique, des infiltrats inflammatoires néoplasiques, des ganglions lymphatiques régionaux drainants et au sein de tout autre site ou liquide biologique accessible de l'être humain à partir duquel les cellules du système immunitaire ont été prélevées auparavant.
3. Méthode de détermination d'une maladie résiduelle minimale chez des patients à risque en mesurant l'activité cytolytique des lymphocytes effecteurs T et NK antitumoraux selon la méthode des revendications 1 et 2.
4. Méthode selon la revendication 3, dans laquelle l'activité cytolytique est mesurée soit par des techniques d'imagerie optique rendues possibles en marquant auparavant aux moins lesdites cellules cibles, en particulier les cellules tumorales et les micro-organismes, soit par des techniques de mesure d'impédance.
5. Méthode selon l'une quelconque des revendications 1 à 4, comprenant en outre une étape de prélève-

ment et d'expansion des cellules du système immunitaire qui ont été sélectionnées à la fin de l'étape c) afin de créer des clones d'effecteurs antitumoraux sélectionnés en amont pour l'efficacité particulière du mécanisme lytique.

6. Méthode selon la revendication 5, dans laquelle la dite étape d'expansion est réalisée par une amplification et une re-sélection *ex vivo* des effecteurs avec des caractéristiques optimales, de façon à éliminer les populations clonales instables, en répétant les étapes a), b) et c) sur les cellules du système immunitaire expansées qui ont été sélectionnées à la fin de l'étape c).
7. Utilisation de la méthode selon l'une quelconque des revendications 1 à 6 pour la préparation d'un médicament comprenant des cellules du système immunitaire qui ont été sélectionnées à l'aide desdites étapes a), b) et c) et/ou leurs clones dans un support approprié pour une re-perfusion *in vivo* pour des objectifs thérapeutiques, éventuellement après une stimulation par des cellules dendritiques.
8. Utilisation selon la revendication 7, pour la préparation d'un médicament destiné au traitement d'une pathologie qui peut être traitée par perfusion de cellules effectrices lytiques sélectionnées et expansées.

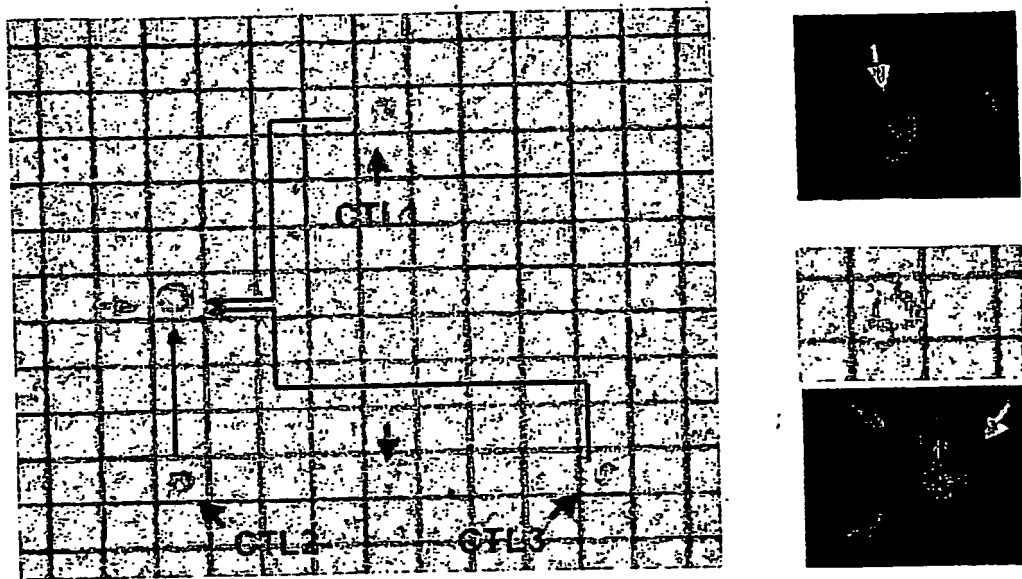


Fig. 1

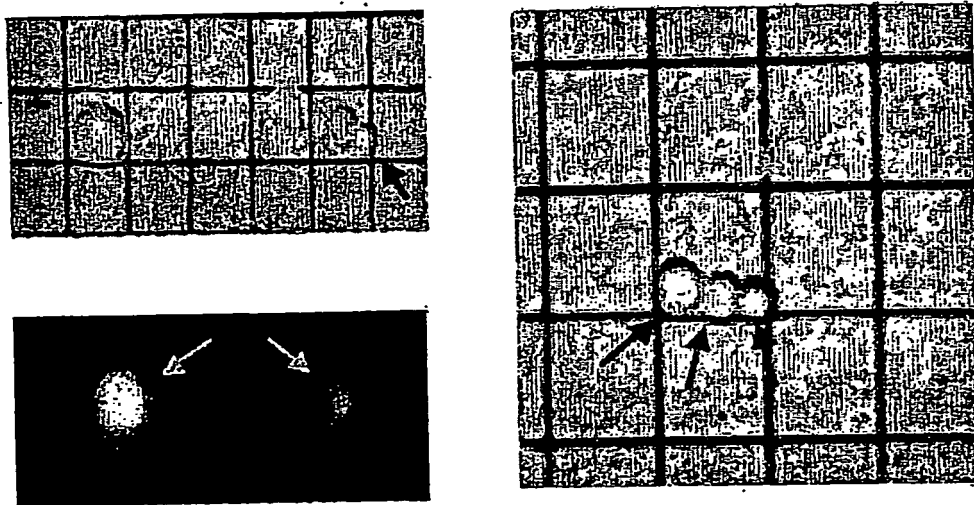


Fig. 2

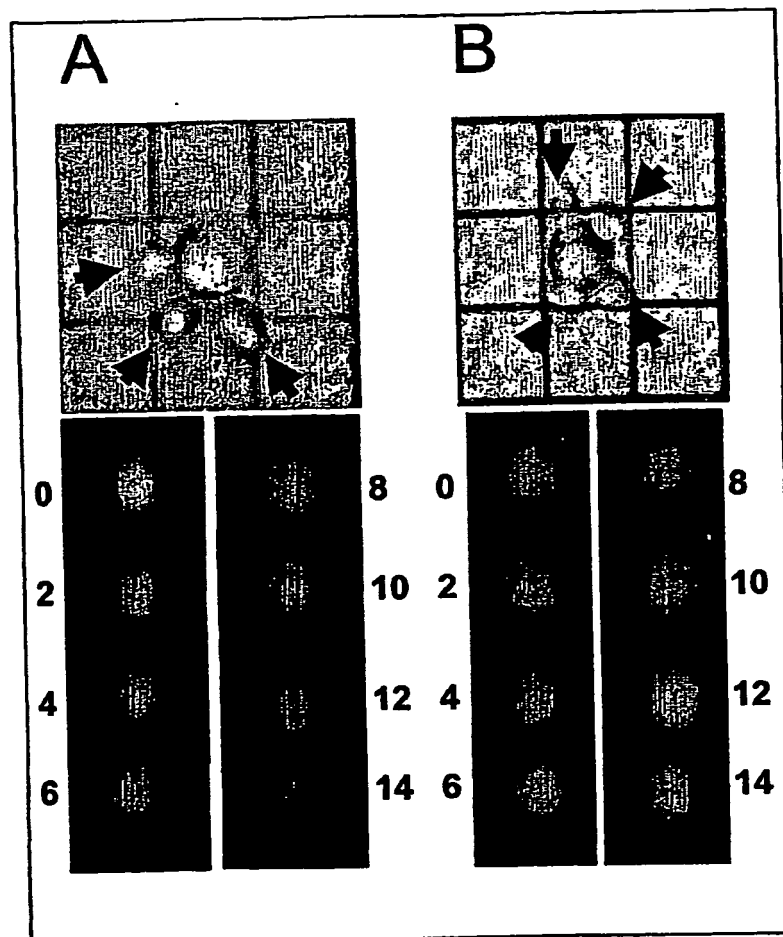


Fig. 3

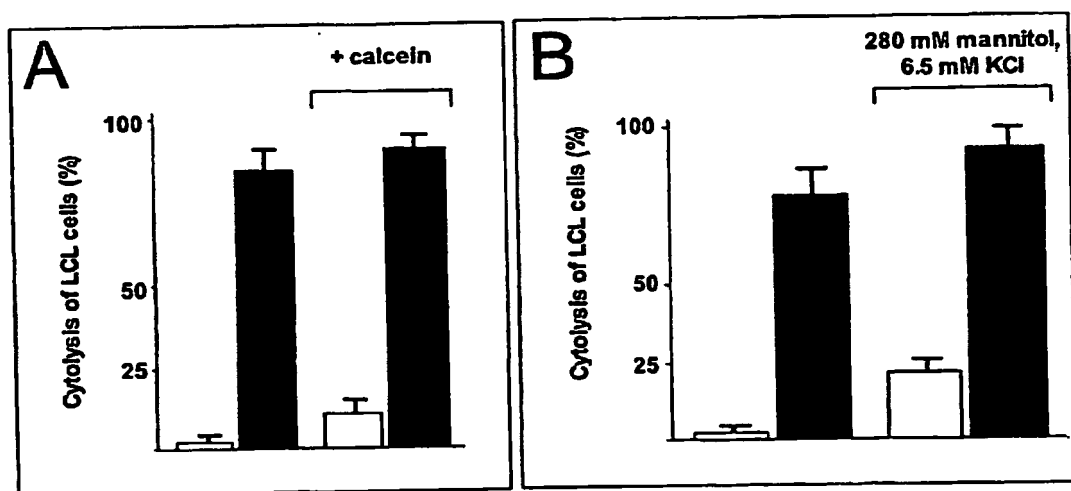


Fig. 4

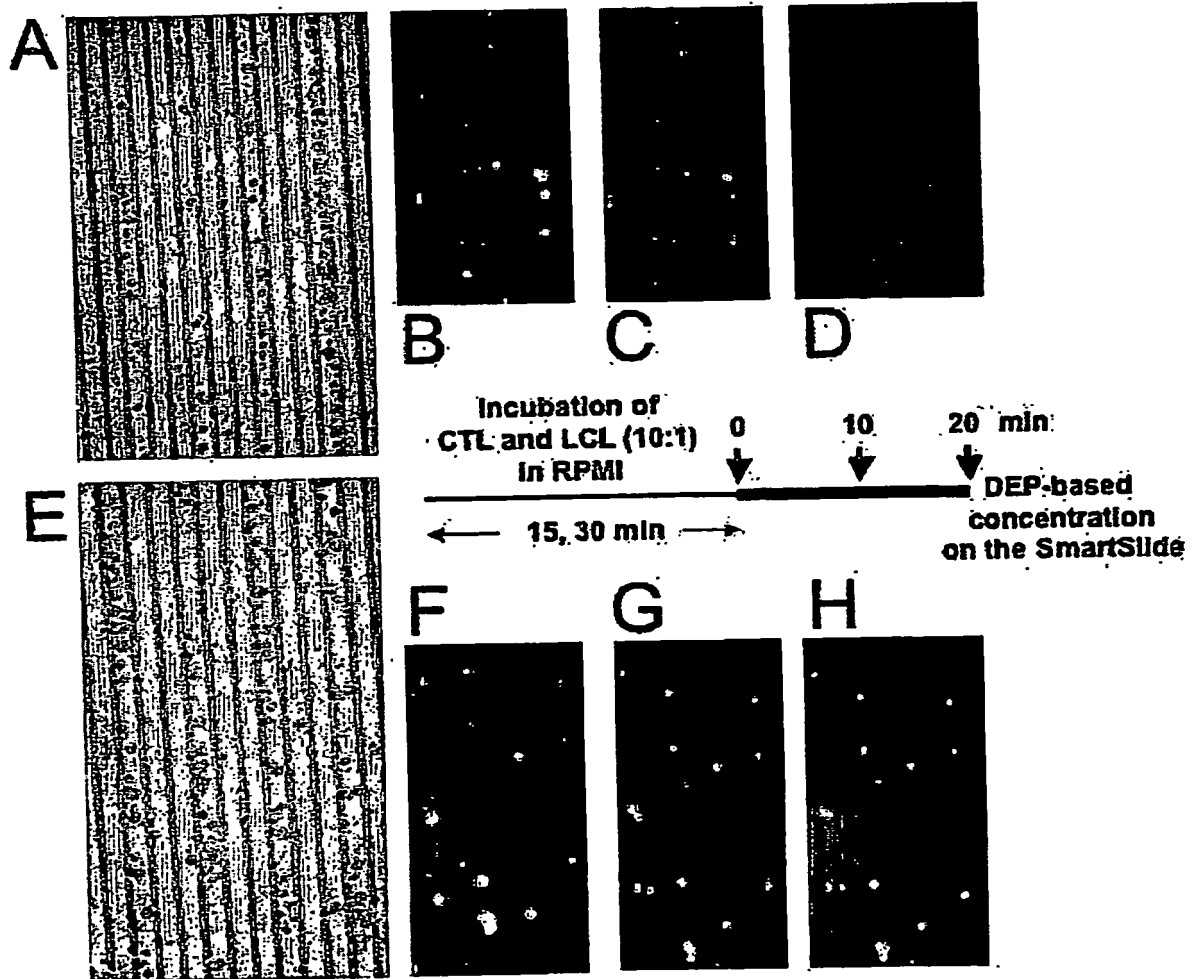


Fig. 5

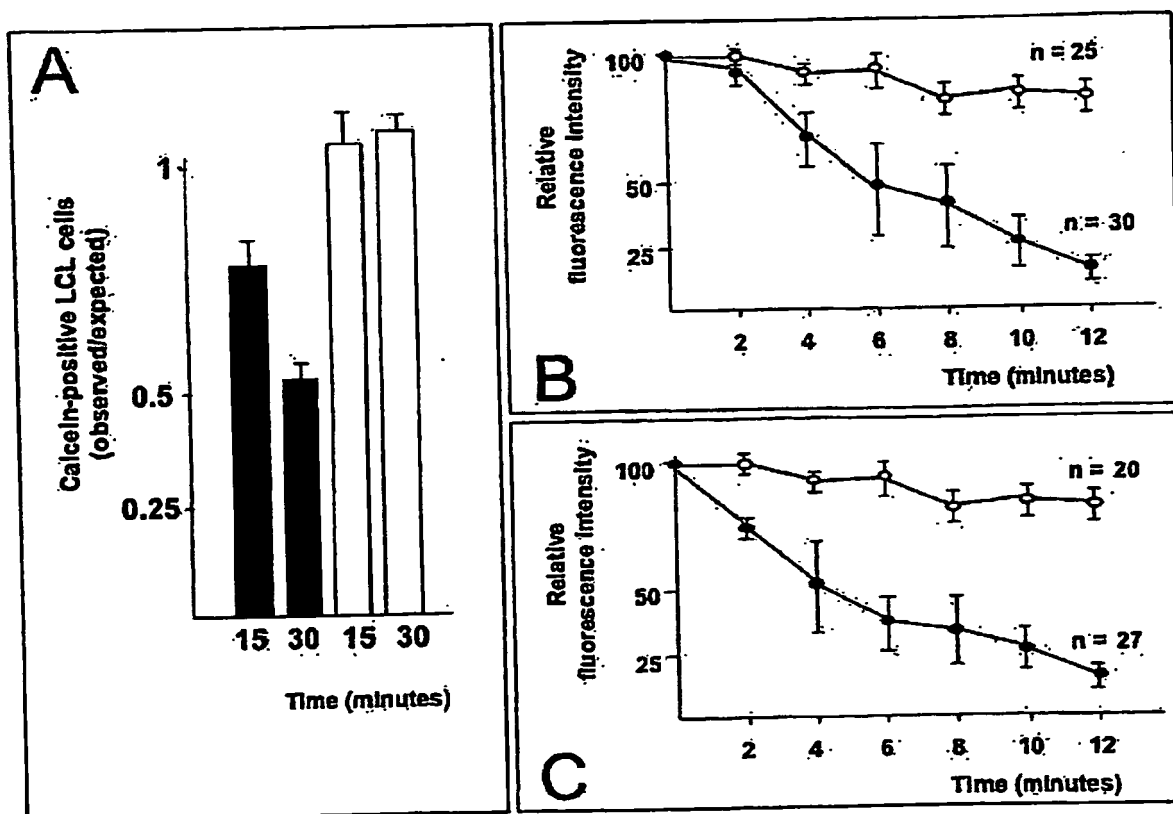


Fig. 6

REFERENCES CITED IN THE DESCRIPTION

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